UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

OFFICE OF CHEMICAL SAFETY
AND POLLUTION PREVENTION

MEMORANDUM

SUBJECT: EPA's Response to Comments Received on the April 26, 2016, Notice of Receipt for an Amendment and Extension to Experimental Use Permit 88877-EUP-2 (Docket ID Number: EPA-HQ-OPP-2015-0374; FRL-9944-96)

FROM: Shannon Borges, Senior Scientist (Acting)
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

Milutin S. Djurickovic, Biologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

Jeannine Kausch, Regulatory Action Leader
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

John L. Kough, Ph.D., Senior Scientist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

Gail Tomimatsu, Ph.D., Plant Pathologist
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

Chris A. Wozniak, Ph.D., Biotechnology Special Assistant
Biopesticides and Pollution Prevention Division (7511P)

THRU: ShaRon Carlisle, Associate Chief
Microbial Pesticides Branch
Biopesticides and Pollution Prevention Division (7511P)

TO: Robert McNally, Director
Biopesticides and Pollution Prevention Division (7511P)

BACKGROUND

On October 15, 2015, the U.S. Environmental Protection Agency (EPA) issued experimental use permit 88877-EUP-2 to the University of Kentucky's Department of Entomology (UKDE). Under experimental use permit 88877-EUP-2, UKDE can release and monitor 2,400,000 male *Aedes aegypti* WB1 Strain mosquitoes that contain the pesticidal active ingredient *Wolbachia pipientis*, *w*AlbB Strain (5.672×10^{-5} ounce of active ingredient in total) in Fresno County, California in 2015 and 2016 over 840 acres. The testing allows UKDE to collect information (e.g., product performance data) that is necessary for it to obtain a pesticide registration under section 3 of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA).

On February 1, 2016, EPA received an application from UKDE to amend and extend experimental use permit 88877-EUP-2 by adding sites in Orange County, California and Monroe County, Florida in 2016 and 2017 and by continuing testing in Fresno County, California in 2017. Up to 12,000,000 additional male *Aedes aegypti* WB1 Strain mosquitoes containing *Wolbachia pipientis*, *w*AlbB Strain are proposed to be released and up to 748.3 additional acres (includes point-source release and surveillance/monitoring acreage) will be involved in testing in 2016 and 2017. EPA determined that this particular application "may be of regional or national significance" ([HYPERLINK "http://www.ecfr.gov/cgi-bin/text-idx?SID=5b676b52e583e20a39cb0702847edb74&mc=true&node=se40.24.172_111&rgn=div8"]); therefore, it published a Notice of Receipt (NOR) in the Federal Register of April 26, 2016 ([HYPERLINK "https://www.gpo.gov/fdsys/pkg/FR-2016-04-26/pdf/2016-09745.pdf"]). In response to this publication, EPA received 11 public comments that consist of a mix of negative, neutral, and positive comments from private citizens, a company (Oxitec, Ltd.), and a non-governmental organization (Center for Food Safety). EPA appreciates all of the comments received, although its primary focus below is to reiterate and respond to the negative and neutral comments.

PUBLIC COMMENTS AND EPA'S RESPONSE

Because some of the comments raised similar issues, EPA grouped similar comments together and generated one response to each grouping. When grouping text from multiple comments, EPA provides specific details as to where the text originates.

PUBLIC COMMENT #1

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0011"] – Please don't release any gmo mosquitoes. You must know that gmo mosquitos released in South America carried piggy back DNA that is probably the cause of the initial mutation. Stop, just stop.

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0014"] – I do NOT AGREE with this plan. We need to stop messing around with nature so much; we are causing our own problems. I do not trust biotech at all . . . they are the "problem, reaction, solution" type of industry. They create a problem, incite reaction and magically come up with a solution. Please do not go forward with this plan.

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0016"] – In regard to EPA-HQ-OPP-2015-0374 (University of Kentucky's Department of Entomology requesting an

amendment and extension to an already existing experimental use permit (EUP) for *Wolbachia pipientis*, *w*AlbB Strain), I would like to have the results of the previous experimental use permit before this extension is analyzed. Why is an extension needed? Did the plan not work, or was the plan unclear, or was the plan poorly performed, etc? I am a graduate engineer working in the Environmental Engineering field. I am not clear on what makes this "new" breed new! If the *Wolbachia* bacteria is being modified, then I think that this should be reviewed by the USDA. I am suspicious that the permit application is being assigned to the FDA in order to avoid the more stringent USDA protocols. The comments submitted to the FDA are much fewer than those submitted to the USDA. Is this an example of Crony Capitalism? Do NOT approve this request because it is not transparent to the public!

EPA'S RESPONSE TO PUBLIC COMMENT #1

Because these comments (and others not reiterated above) cite to genetically modified mosquitoes (which the mosquitos that are subject to this EUP are not) or other agencies besides EPA, and because another comment ([HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0012"],) quoted in full under Public Comment #3, below, incorrectly associates the *Wolbachia* that is the subject of this EUP with reducing "the susceptibility of the insect [mosquito] to infection by Zika and other viruses," EPA believes that the differences between the three technologies that work through release of live mosquitoes are being conflated. For purposes of clarification, therefore, brief explanations of these three technologies follow below.

(1) *Wolbachia pipientis*-infected mosquitoes intended to suppress populations of particular species of mosquitoes. The *Wolbachia pipientis*-infected mosquitoes released by UKDE during experimental use permit 88877-EUP-2 are intended to suppress populations of *Aedes aegypti*. As *Wolbachia pipientis* generally does not occur in natural populations of *Aedes aegypti*, the *w*AlbB Strain of *Wolbachia pipientis* is extracted from *Aedes albopictus* embryos and microinjected into *Aedes aegypti* embryos. These mosquitoes are reared in isolated containment facilities that follow Arthropod Containment Level-1 procedures (Tabachnick, 2006). Polymerase chain reaction (PCR) is used to confirm the presence of the *w*AlbB Strain in the infected mosquitoes. Male mosquitoes are separated from female mosquitoes and shipped to testing sites where they are released and mate with wild-type *Aedes aegypti* females that do not carry *Wolbachia*. Through cytoplasmic incompatibility, the embryos from these matings die and the eggs do not hatch (Weeks, 2015). These mosquitoes and the microbes within them are not genetically modified/engineered, and the *Wolbachia pipientis* in these mosquitoes is regulated as a microbial pesticide by EPA under FIFRA (U.S. EPA, 2013). EPA issued, amended, and/or extended other experimental use permits using this same technology in *Aedes polynesiensis* or *Aedes albopictus* in 2012, 2013, and 2014.[1]

(2) Genetically engineered mosquitoes intended to suppress populations of particular species of mosquitoes. Oxitec OX513A *Aedes aegypti* are engineered to produce a lethal protein that is only expressed in the absence of tetracycline, an antibiotic. These mosquitoes can reproduce generation after generation in the laboratory in the presence of tetracycline, but larvae in the wild cannot mature and die in the absence of tetracycline. A red fluorescent marker protein, which is also expressed in the mosquitoes, is used for quality control and monitoring in the field. Male Oxitec OX153A mosquitoes are released into the environment and mate with wild-type *Aedes aegypti* females, thereby passing the genes that produce the lethal and marker proteins to their offspring. These mosquitoes are genetically

[1] See docket number EPA-HQ-OPP-2012-0181 and docket number EPA-HQ-OPP-2013-0254 at [HYPERLINK "http://www.regulations.gov"].

modified/engineered and are regulated as a new animal drug by the U.S. Food and Drug Administration (FDA) under the Federal Food, Drug, and Cosmetic Act (FFDCA) (U.S. EPA, 2013).[2]

(3) *Wolbachia pipientis*-infected mosquitoes intended to reduce the capacity of mosquitoes to harbor and transmit arboviruses (e.g., dengue and Zika viruses). Certain strains of *Wolbachia pipientis* (e.g., *w*Mel and *w*MelPop) introduced into *Aedes aegypti* reduce the ability of the mosquitoes to transmit harmful human viruses like dengue (Ye et al., 2015) and Zika (Aliota et al., 2016). To establish mosquitoes with these *Wolbachia* strains in the environment, both male and female mosquitoes (not just male mosquitoes as described in #1 and #2 above) must be released. Since 2011, the Eliminate Dengue research program, a not-for-profit international collaboration led by Monash University, has been conducting small-scale field trials in Australia and subsequently in Vietnam, Indonesia, Brazil, and Colombia with *Wolbachia pipientis*-infected mosquitoes. These mosquitoes and the microbes within them are not genetically modified/engineered.[3]

Regarding the questions about the experimental use permit, EPA allows for extensions and amendments to these programs in accordance with [HYPERLINK "http://www.ecfr.gov/cgi-bin/text-idx?SID=b4aa74998fdad9e2fa2c53baedfc6998&mc=true&node=se40.26.172_15&rgn=div8"] if circumstances warrant. In 2015, EPA permitted UKDE to conduct testing for approximately one year in Fresno County, California. In order to collect more information necessary to support an application for pesticide registration, UKDE requested to extend the testing another year and include sites in two additional counties in the United States. As explained below in response to other comments, EPA believes UKDE's request is justified and all other requirements have been met to allow extension and amendment of experimental use permit 88877-EUP-2. UKDE will submit a final report, which includes all data gathered during the experimental program, to EPA upon conclusion of testing. More information on experimental use permits can be found in [HYPERLINK "https://www.epa.gov/pesticide-registration/pesticide-registration-manual-chapter-12-applying-experimental-use-permit"].

PUBLIC COMMENT #2

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0017"] – I was … shocked to discover that application 88877-EUP-2 from the University of Kentucky, Department of Entomology was not available for examination and that all of the information available for public comment was that the application sought "to amend and extend this EUP by adding sites in Orange County, California and Monroe County, Florida (Florida Keys) in 2016 and 2017 and by continuing testing in Fresno County, California in 2017. Up to 12,000,000 additional male *Aedes aegypti* WB1 Strain mosquitoes containing *Wolbachia pipientis*, *w*AlbB Strain (28.36 Å~ 10¥5 ounce) are proposed to be released and up to 748.3 additional acres (includes point-source release and surveillance/monitoring acreage) will be involved in testing in 2016 and 2017. The released male mosquitoes are expected to mate with indigenous female mosquitoes, causing conditional sterility and resulting in population decline and potential elimination. Adult and egg collection data from the treated areas will be compared to data from control sites to evaluate the effect of the pesticide on mosquito populations."

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[2] See docket number FDA-2014-N-2235 at [HYPERLINK "http://www.regulations.gov"] or see U.S. FDA (2016) for more information on these mosquitoes.

[3] See [HYPERLINK "http://www.eliminatedengue.com/program"] for more information on these mosquitoes.

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0018"] – Oxitec submits these comments to strongly convey that prior to approval of the uncontained release to the environment of substantial numbers of modified mosquitoes carrying this strain of *Wolbachia pipientis* EPA must conduct a sufficiently rigorous assessment of the potential adverse impacts to human health and the environment that such uncontained release of modified mosquitoes may entail. As we discuss in detail below, Oxitec believes that there are significant questions regarding these potential impacts and these must be addressed prior to any regulatory approval.

Moreover, it is not clear from the public record of this matter if sufficient data and information have been submitted by the applicant to address these issues. The information available in public docket EPA-HQ-OPP-2015-0374 for the *Wolbachia* EUP application 88877-EUP-2 is extremely limited. The paucity of information available in the docket makes it difficult for the public to comment on whether the application should be granted, or what should be the scope of any potential approval of the EUP. For the public in general, and Oxitec in particular, to provide meaningful comment on whether this application to amend and extend the EUP should be granted, more information relevant to a determination of environmental and human safety should be available as part of the public record. Moreover, should EPA determine after review to grant the application for amendment and extension of the EUP, EPA must make available all data and information supporting such decision, and clearly explain how the critical human health and ecological issues raised in these comments are addressed.

At the outset, we note that Oxitec has developed a different genetic insect control technology that has been demonstrated to be efficacious in significantly reducing the population of disease-carrying mosquitoes (>90% in the Cayman Islands, Brazil, and Panama). Because Oxitec's insect control technology utilises genetic engineering of the insect genome, it has been determined that it is to be regulated by the U.S. Food and Drug Administration as a new animal drug. It is the oft-stated policy of the U.S. government that it regulates the products of biotechnology on the basis of a "risk-based, scientifically sound approach . . . that focuses on the characteristics of the biotechnology product and the environment into which it is being introduced, not the process by which the product is created." *Exercise of Federal Oversight Within Scope of Statutory Authority; Planned Introductions of Biotechnology Products Into the Environment*, 57 Fed. Reg. 6753 (Feb. 27, 1992) (this seminal statement is repeated throughout the Policy Statement, see, e.g., *Id.* at 6754-55, 6755, 6756, 6757, and 6760). Notwithstanding this consistently stated position, the reality is that Oxitec's self-limiting mosquitoes have been subjected to a mandatory pre-market approval regulatory process at FDA that has been much more onerous than the regulatory requirements faced by the *Wolbachia pipientis* microbial pesticide at EPA. The distinction between the EPA review process for *Wolbachia*, and that faced by Oxitec's self-limiting mosquitoes has even been noted by *Nature* [Waltz, 2016].

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Notwithstanding this potential for indiscriminate gene transfer, the *Wolbachia* IIT (Incompatible Insect Technique) vector control method is subjected to a substantially less rigorous regulatory review process than is a targeted genetic engineering methodology. This is directly contrary to the intent of the Coordinated Framework and to a scientifically valid risk-based regulatory process.

Such a disparate regulatory approach to two products intent on achieving similar public health ends is inconsistent with the stated Federal regulatory policy, and, as a matter of risk-based regulatory process, is without reason and justification. Oxitec's self-limiting technology and the *Wolbachia* IIT approach both have the intended purpose of end point reductions in the population of mosquitoes and involve

releases of substantial numbers of non-wild type mosquitoes to the environment. . . . Therefore, as a matter of sound regulatory action, it is incongruous for the regulatory burden placed on the *Wolbachia* IIT technology to be significantly less onerous and burdensome than the requirements imposed on Oxitec's self-limiting targeted genetic engineering technology.

As an example of the disparate regulatory treatment that Oxitec's self-limiting mosquitoes have faced, FDA's Center for Veterinary Medicine (CVM) established an Animal Biotechnology Interdisciplinary Group (ABIG) to evaluate Oxitec's technology. This ABIG included experts from FDA/CVM, CDC, and EPA. It is not clear from the record if the UKDE EUP application was shared with other regulatory agencies and if they were given a chance to comment. In this regard, we believe that the FDA review process for Oxitec's self-limiting technology raised questions and considerations that are relevant to application 88877-EUP-2 to amend and extend the current *Wolbachia* EUP.

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Based on the available record, it is not clear what level of environmental assessment has been conducted to this point. Because *Wolbachia* has been demonstrated to affect insects in the environment – changing their behaviour, disease transmission status, gene expression and biology [Endersby and Hoffmann, 2013; Werren et al., 2008; Marshall, 2007] – Oxitec believes that approval of widespread release of this modified organism without a comprehensive ecological risk assessment is wholly inconsistent with EPA's statutory and regulatory responsibilities under FIFRA.

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In conclusion, Oxitec requests that EPA make publicly available for comment the data and information on which it bases its regulatory decision on the UKDE EUP application, and explain the basis for that decision. Moreover, consistent with FIFRA Section 5(d), EPA should, prior to this strain of *Wolbachia pipientis* being considered for registration and consistent with the serious concerns identified above, specify to the applicant specific data and information that must be provided to address the ecological and human health concerns identified in these comments regarding unreasonable adverse effects on the environment.

EPA'S RESPONSE TO PUBLIC COMMENT #2

The *Aedes aegypti* OX513A Release of Insects with Dominant Lethality (RIDL) technology is unique in its approach to mosquito population suppression and relies on a novel antibiotic sensitive, transcriptional activator mechanism genetically engineered into *Aedes aegypti* in order to effect lethality of insects following release into the environment and the absence of tetracycline (as present under laboratory conditions). The *Wolbachia*-based *Aedes aegypti* product proposed for experimental field trials mimics the cytoplasmic incompatibility phenotype as known from numerous insects and other arthropods; while a single report exists in the literature indicating natural infection of *Aedes aegypti*, it is estimated that greater than 1 million species extant in the environment harbor naturally occurring *Wolbachia* strains. This presence and degree of exposure to a variety of organisms without documented negative impacts suggests that the product under consideration by EPA is also likely to pose minimal probability of adverse effects to humans and the environment.

The Coordinated Framework for the Regulation of Biotechnology, as proposed in 1986, came about because of the advent of new recombinant DNA techniques that resulted in the directed engineering of

organisms to achieve phenotypes not previously possible with conventional techniques ([HYPERLINK "https://www.aphis.usda.gov/brs/fedregister/coordinated_framework.pdf"]). In the 1992 update to the Coordinated Framework ([HYPERLINK "https://www.whitehouse.gov/sites/default/files/microsites/ostp/57_fed_reg_6753__1992.pdf"]), the principles outlined therein and their specific implementation must be developed within the context of each agency's statutory programs.

As explained in EPA's May 20, 2013, response to comments regarding an experimental use permit for the release of *Aedes polynesiensis* mosquitoes infected with a novel strain of *Wolbachia pipientis* (U.S. EPA, 2013):

> EPA has jurisdiction over the *Wolbachia pipientis* bacteria that are the subject of this EUP because such bacteria constitute a substance intended for preventing, destroying or mitigating a pest, and therefore meet the definition of "pesticide" under Section 2(u) of the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). … In contrast, because the recombinant DNA (rDNA) construct in the genetically engineered mosquitoes developed by Oxitec using RIDL technology is being regulated by the U.S. Food and Drug Administration (FDA) as a "new animal drug" under the Federal Food, Drug, and Cosmetic Act (FFDCA), such technology does not meet the definition of "pesticide" under section 2(u) of FIFRA, which states, in part, that "… the term 'pesticide' shall not include any article that is a 'new animal drug' within the meaning of section 321(w) of Title 21…."

The type or quantity of information requested by FDA in its analysis of the OX513A product as a "new animal drug" under the FFDCA is a matter for FDA, not EPA. EPA is properly granting the present Amendment and Extension to Experimental Use Permit 88877-EUP-2 pursuant to section 5 of FIFRA and [HYPERLINK "http://www.ecfr.gov/cgi-bin/text-idx?SID=20d021706b509f7011a812e7b77c4072&mc=true&node=pt40.26.172&rgn=div5"].

[HYPERLINK "http://www.ecfr.gov/cgi-bin/text-idx?SID=20d021706b509f7011a812e7b77c4072&mc=true&node=se40.26.172_111&rgn=div8"] specifies the information that must be included in a notice of receipt (NOR) of an application, published in the Federal Register, for an EUP if EPA finds that issuance of the EUP may be of regional or national significance. EPA published the NOR for the present EUP action, containing all the required information, on April 26, 2016 ([HYPERLINK "https://www.gpo.gov/fdsys/pkg/FR-2016-04-26/pdf/2016-09745.pdf"]). The product characterization, toxicological, and nontarget organism considerations as reviewed by EPA are now contained in the docket ([HYPERLINK "https://www.regulations.gov/docket?D=EPA-HQ-OPP-2015-0374"]). EPA believes its information request and review adequately assess any human health or environmental concerns potentially posed by this limited release of *Aedes aegypti w*AlbB mosquitoes. As part of the approval of an experimental use permit for such a release, UKDE is required to continue to collect specific information and transmit this to EPA in a timely manner. As with all pesticides, UKDE is also required, under section 6(a)(2) of FIFRA, to report to EPA any factual information regarding unreasonable adverse effects on the environment noted during testing and use of a pesticide product. Accordingly, EPA does not see a need in this instance and at this time to exercise the discretionary authority provided under FIFRA section 5(d).

PUBLIC COMMENT #3

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0012"] – Zika is one of several viruses [dengue, yellow fever, chikungunya] transmitted by *Aedes aegypti*, a mosquito that is common in urban areas throughout much of the tropics. At present, there is no evidence that any of the conventional control methods used to combat this mosquito has any impact on transmission; the meteoric rise of dengue in the past few decades, followed by chikungunya and now Zika, is testament to the inefficacy of these methods. There is therefore an urgent need for new and innovative approaches. To my mind, as a specialist in this field the most promising of these, at present, is the transgenic produced by the UK company Oxitec. A second interesting approach involves the release of male insects infected with the bacterial intra-cellular parasite, *Wolbachia*, which, in theory, should be able to irrevocably infect a wild population of the mosquito in the field and thereby reduce the susceptibility of the insect to infection by Zika and other viruses.

I am glad that the EPA is considering a further permit for field releases but I am surprised that so little information is presented on aspects such as vector competence of the infected insect.

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0017"] – The use of *Wolbachia* to control mosquito populations is in its experimental infancy. The basic observations are that 1) *Wolbachia* infection spreads and takes over an insect population by a variety of mechanisms and 2) that *Wolbachia*-infected insects show reduced transmission of some diseases, including malaria and dengue. However, this is far from universal, and indeed, there is evidence that *Wolbachia* can increase the titer of the pathogen in an insect host (Hughes et al., 2014[a]; Dodson et al., 2014).

The wording available to the public indicates that the release of *Wolbachia*-infected insects has the objective of suppressing the population through induction of sterility. It is noted that the University of Kentucky will release only males. However, the females are not genetically sterile, hence sorting is most likely done by size and visual inspection, neither of which is 100% effective. Hence the most straightforward long-term outcome will be that the introduced strain replaces the native strain through amplification of the offspring of matings between *Wolbachia*-infected males and females, however infrequent. Suppression will likely be short-term and simple takeover of the population by *Wolbachia*-infected *A. aegypti* will be the long-term outcome.

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0018"] – What is the likelihood that *Wolbachia* mosquitoes can reproduce and establish in the environment into which they are released?

Wolbachia is a bacterium residing within the cells of insects, and is passed through vertical transmission from mother to offspring. Even a single *Wolbachia* infected female could lay hundreds of eggs that would invade the wild population, rendering the Incompatible Insect Technique ineffective and spreading a new strain of *Wolbachia* into the environment. Modelling has shown that conditions of lower competition can favour infected females [Endersby and Hoffmann, 2013; Hancock et al., 2011; Jansen et al., 2008]. In other words, as a mosquito population is reduced, or if a population is already low, the chances of *Wolbachia* invading the wild population are increased.

Given that the release of females carrying the *Wolbachia* strain in the cytoplasm is to be avoided, sex sorting should approach 100%. Yet, studies have shown that mechanical sex sorting with IIT [Incompatible Insect Technique] is only 90% effective in removing females. Thus, the reported

inefficiency of sex sorting of *Aedes aegypti* mosquitoes infected with *Wolbachia* presents a risk that must be evaluated [O'Connor et al., 2012].

Any unexpected effects of *Wolbachia* could persist in the wild if any females are released, with little possibility of recall [Alphey, 2009].

According to the proposed EUP label: "Only male (and no female) *Ae. aegypti* strain WB1 mosquitoes carrying *Wolbachia pipientis*, wAlbB Strain microbial pesticide are to be released." We question whether this statement is correct. No information has been publicly provided to demonstrate that the sorting methods used to generate populations of mosquitoes to be released guarantee that females will not be released. Nor has information been provided regarding quality control monitoring that will be conducted to ensure this.

The permit requires the user to 'sample released mosquitoes to confirm the rate of female mosquitoes released'. But nowhere does it say how this will be done. As this is such an important aspect of the safety and efficacy of the system any EUP that allows release of *Wolbachia* mosquitoes should be revoked and releases stopped if females are detected.

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Moreover, the possible persistence of *Wolbachia* mosquitoes themselves is a significant concern. For the reasons set forth below, each new strain of mosquito, or indeed any artificially *Wolbachia* infected insect needs to be treated as a new strain and thoroughly tested in the laboratory before any field releases.

Aedes aegypti artificially modified with *Wolbachia* show a reduction in dengue virus replication but virus is still found in the saliva of these engineered mosquitoes which therefore have the capacity, even if reduced, to transmit disease [Ye et al., 2015].

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Temperature impacts *Wolbachia*-malaria interaction in mosquitoes suggesting impact of transfection might vary across diverse environments [Murdock et al., 2014].

Research has shown *Wolbachia* enhances West Nile virus infection in the mosquito *Culex tarsalis*. This introduces the possibility that the *Wolbachia* infection could spread to *Culex* populations in areas where West Nile virus is a concern [Dodson et al., 2014].

Research has shown *Wolbachia* can enhance malaria parasite infection in two genera of mosquitoes [Hughes et al., 2014a; Hughes et al., 2012; Zele et al., 2014].

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The release of any females as a result of mis-sorting for any reason would allow the *Wolbachia* strain to invade the wild populations of mosquitoes in the release area, thus rendering this control approach ineffective and with the potential to spread a new strain of *Wolbachia* into the environment with unknown outcomes and consequences.

EPA'S RESPONSE TO PUBLIC COMMENT #3

The *w*AlbB *Aedes aegypti* strain is not intended to affect the competency of the vector to transmit viral agents. Currently, there is no compelling evidence that *w*AlbB in *Aedes aegypti* does affect the capacity of the vector to transmit disease agents. A few published manuscripts have discussed West Nile virus (WNV) and *Plasmodium* titers increasing in *Wolbachia*-positive strains, but *Aedes aegypti* are not a natural malaria vector and *Aedes aegypti* do not generally carry WNV (Hughes et al., 2014a). Because *Aedes aegypti* are not a natural malaria vector, research showing the effects in *Culex pipiens* cannot be used to assume this is true for *Aedes aegypti*. Additionally, Hughes et al. (2012) discusses *Plasmodium* infections in *Anopheles gambiae* with *w*AlbB, and this is not applicable to the situation with *Aedes aegypti*. Furthermore, the manuscript that discusses the increase in WNV titer in *w*AlbB-infected *Culex tarsalis* (Dodson et al., 2014) resulted from a somatic *Wolbachia* infection, which cannot be considered the same as a stably inherited *Wolbachia* infection through vertical transmission.

It may be possible to establish a *w*AlbB *Aedes aegypti* population with use of the *w*AlbB strain. This, however, would only be possible if substantial numbers of females were released into an ecosystem along with repeated male *w*AlbB *Aedes aegypti* releases. The release of females is strictly controlled in the quality control procedures during mechanical separation of pupae and microscopic inspection of sorted pupae (U.S. EPA, 2015a). In addition, UKDE monitors the environment near its rearing facilities for inadvertent release or escape of female *Aedes aegypti w*AlbB. As with any mechanical separation technique for mosquitoes, continual monitoring and quality assurance measures are paramount for ensuring that only males are released.

The Eliminate Dengue program (described above in Response to Public Comment #1, above) has achieved replacement of wild *Aedes aegypti* populations with *Wolbachia*-infected populations through substantial repeated releases of both high numbers of males and females. Even with this strategy of deliberate male and female releases, failure to establish *Wolbachia*-infected populations has occurred in some instances. Establishing a *w*AlbB *Aedes aegypti* population is highly unlikely because only males are released with very few potential accidental female releases, i.e., less than 1 female per 250,000 males (U.S. EPA, 2015a and 2015b). According to Xi et al. (2005), in caged releases of *w*AlbB *Aedes aegypti* females with uninfected males, a minimum of 20% of females needed to be released to establish the *Wolbachia* infection after seven generations. All releases below 20% in that cage experiment resulted in failure of the *w*AlbB infection to be established in the population.

PUBLIC COMMENT #4

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0017"] – Moreover, integration of the entire *Wolbachia* genome into the insect's genome has been reported (Nikoh et al., 2008). Furthermore, *Wolbachia* infection has been shown to cause changes in reproductive structure in wild-type insect populations (Marshall, 2007).

In view of these observations, both an environmental impact assessment [EIA] and data on the ability of the *Wolbachia*-infected population to acquire and transmit relevant diseases, including dengue, West Nile Virus, and particularly the Zika virus, are essential. Yet it appears that the EPA is about to approve release of large numbers of infected insects without either an EIA, studies on its safety, or a back-up plan should the population be fully converted *Wolbachia*-infected or should unexpected adverse effects surface.

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0018"] – As is detailed below, horizontal gene transfer could result in *Wolbachia* effectively introducing over one thousand new genes into the recipient organism.

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Importantly, however, Oxitec's self-limiting genetic engineering is well-defined and includes only two well-studied genes, which were purposefully added and reviewed by the regulatory agencies, whereas the potential genetic modification that may result from use of wAlbB *Wolbachia* bacterium is wholly undefined. Several studies have shown that horizontal gene transfer between *Wolbachia* and their insect hosts may result in gene transfers ranging from nearly the entire *Wolbachia* genome (>1 megabase) to short insertions (<500 base pairs) into various hosts [Dunning Hotopp et al., 2007; Fenn et al., 2006; Hou et al., 2014; Kondo et al., 2002]. Effectively the *Wolbachia* IIT approach could introduce over one thousand new genes into the target mosquito with unknown consequences, and if they provide a positive selection to the mosquito in the environment this could result in novel strains pervading and spreading through the population.

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The *Wolbachia* is an endosymbiont on the cytoplasm of the cell so over a thousand new genes are introduced into the insect cells, some or all of which have the potential to randomly integrate into the insect's nuclear genome with unknown consequences. Moreover, the possible persistence of *Wolbachia* mosquitoes themselves is a significant concern. For the reasons set forth below, each new strain of mosquito, or indeed any artificially *Wolbachia* infected insect needs to be treated as a new strain and thoroughly tested in the laboratory before any field releases.

. . . .

The whole genome of *Wolbachia* can transfer to a host genome, meaning a host mosquito could be transformed with over one thousand new genes with unpredictable results [Dunning Hotopp et al., 2007; Fenn et al., 2006; Hou et al., 2014; Kondo et al., 2002].

It has already been shown that horizontal gene transfer (HGT) can transfer genes between *Wolbachia* and its host in *Aedes aegypti* [Klasson et al., 2009] and several other mosquito species [Woolfit et al., 2009]. Therefore, *Wolbachia* can genetically transform its host with functional genes with currently unknown consequences.

Widespread recombination occurs throughout the *Wolbachia* genome [Baldo et al., 2006], increasing the likelihood of genes changing as the *Wolbachia* evolves. In addition *Wolbachia* has been shown to change its phenotypic effects on the host insect as it evolves [Carrington et al., 2010]. This could potentially change how *Wolbachia* responds to a number of factors, including how it influences host immune response and vectorial status. Therefore, potentially the vectorial capacity of *Aedes aegypti* infected with *Wolbachia* could change over time and should be continually assessed.

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Introduction of *Wolbachia* into a mosquito provides the possibility to introduce over time over one thousand new genes, yet *Wolbachia* mosquitoes have not been subjected to the rigorous regulatory scrutiny that appears to be the norm for recombinant genetic modification (notwithstanding that typical genetic modification is a 'rifle shot' approach, involving very few, fully characterised, introduced genes). As noted above, this seems entirely incongruent with the stated policy of the U.S. government as set forth in the Coordinated Framework. How can it be ensured that introduction of genes of unknown function are not transferred into the *Aedes aegypti* genome and how will this be tested and monitored through time?

EPA'S RESPONSE TO PUBLIC COMMENT #4

With respect to the integration of the entire *Wolbachia* genome into *w*AlbB *Aedes aegypti* mosquitoes, integration is expected to only occur at long-term evolutionary time scales, if it occurs at all. These events have occasionally occurred in nature, and any integration of *Wolbachia* genes into mosquitoes or mosquito genes into *Wolbachia* are not expected to be different from what has already occurred naturally with *Wolbachia* infection in a million or more arthropod species. No known negative events have been associated with these Horizontal Gene Transfer (HGT) events in nature. While *Wolbachia* can cause male killing, feminization of males, parthenogenesis, and cytoplasmic incompatibility, only the cytoplasmic incompatibility phenomenon has been associated with the *w*AlbB Strain mosquitoes.

The comment referring to an "environmental impact assessment" and "EIA" appears to intend to refer to either an "Environmental Assessment" (EA) or an "Environmental Impact Statement" (EIS) under the National Environmental Policy Act (NEPA). EPA is not required to perform a NEPA analysis when issuing registrations under FIFRA. *Merrell v. Thomas*, 807 F.2d 776 (9th Cir., 1986). However, EPA does conduct an environmental risk assessment for pesticide actions under FIFRA, which the Court in *Merrell* found to be equivalent to an analysis under NEPA. In the present case, EPA conducted an environmental risk assessment for the release of *w*AlbB Strain *Wolbachia*-infected male *Aedes aegypti* mosquitoes and found that adverse effects to nontarget organisms are not expected as a result of the experiments to be performed under this EUP or as a result of its amendment and extension (U.S. EPA, 2015c and 2016).

The presence of the *w*AlbB Strain of *Wolbachia* in *Aedes aegypti* male mosquitoes is not expected to influence vector competence, and the mosquitoes reared for release are evaluated through PCR-based testing for the presence of arboviruses and other pathogens.

PUBLIC COMMENT #5

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0018"] – Can the *Wolbachia* mosquito escape the confined conditions in which it is reared?

What is the likelihood that the *Wolbachia* mosquitoes will survive and disperse once released into the environment?

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What are the potential impacts of the *Wolbachia* mosquitoes in the environment, including on humans?

What are the likely consequences for the surrounding environment, should the *Wolbachia* mosquitoes survive and establish in the environment?

EPA'S RESPONSE TO PUBLIC COMMENT #5

*w*AlbB *Aedes aegypti* mosquitoes are raised in the appropriate arthropod containment facilities (U.S. EPA, 2015a), and according to the UKDE no escaped mosquitoes have been detected from the UKDE rearing facility following more than a decade of monitoring.

The likelihood of *w*AlbB *Aedes aegypti* survival is considered low given that males are dead end hosts, the small number of potential accidental female releases, and bidirectional cytoplasmic incompatibility. Survival would only occur between *w*AlbB male and *w*AlbB female matings in a female accidental release event. As mentioned in the Response to Public Comment #3, at least 20% of the population of females would need to be released to establish a *w*AlbB *Aedes aegypti* constant population. Furthermore, the fitness cost of the *w*AlbB Strain is expected to make the *w*AlbB *Aedes aegypti* mosquitoes less viable than the wild-type population. Accidentally released females could bite a person, but this is not expected to have negative consequences because mosquitoes that are naturally infected with *Wolbachia* bite humans and no negative effects have been reported. Additionally, no antibodies are detected in relation to *Wolbachia* when *Wolbachia*-infected mosquitoes bite humans; *Wolbachia pipientis* has no known history of infecting mammals or other vertebrates (Werren et al., 2008).

EPA anticipates beneficial impact of *Wolbachia*-infected mosquitoes on nontarget vertebrate organisms, including humans, as these pesticides are intended for suppression of mosquito vectors of viral diseases that threaten public health. Adverse impacts are not expected for humans or other vertebrates because strains of *Wolbachia pipientis* are known to establish endosymbiotic relationships only with invertebrates. Because these strains of *Wolbachia pipientis* are naturally occurring, there is already a significant history of exposure to these microorganisms, and information from the open peer-reviewed scientific literature indicates that *Wolbachia* establishes endosymbioses (some mutualistic) with approximately 60% of the insect species, as well as other invertebrates.

EPA anticipates minimal or transient impact on nontarget invertebrate organisms, including nontarget mosquitoes, some of which may be beneficial, owing to the complex endosymbioses and the commonality of this bacterium in invertebrate populations. Adverse impacts are expected to the majority of targeted mosquito populations of *Aedes aegypti*, if naturally occurring females of these species successfully breed with male mosquitoes that have been infected with *Wolbachia pipientis* Strain *w*AlbB.

PUBLIC COMMENT #6

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0018"] – *Aedes aegypti* is not naturally infected with any *Wolbachia* strain, therefore this is an invasive infection in this species.

EPA'S RESPONSE TO PUBLIC COMMENT #6

There is one report of *Aedes aegypti* having a *Wolbachia* strain naturally (NBCI, Undated). *Wolbachia pipientis* is also known to infect approximately 60% of insects and it has been found in other arthropods as well, including some that vector human parasites and pathogens (Ono et al., 2001; Espino et al., 2009). Some of the infections with *Wolbachia* appear to have occurred over evolutionary time to related arthropod species; these are also examples of invasive infections and they are quite common with estimates of greater than a million species being infected to date.

PUBLIC COMMENT #7

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0018"] – For the reasons set forth below, each new strain of mosquito, or indeed any artificially *Wolbachia* infected insect needs to be treated as a new strain and thoroughly tested in the laboratory before any field releases.

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Research has shown the mosquito's microbiome can impede vertical transmission of *Wolbachia* [Hughes et al., 2014b].

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Further work is needed to define the underlying molecular mechanisms of *Wolbachia* induced reproductive modifications, particularly cytoplasmic incompatibility [Brelsfoard and Dobson, 2009].

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Moreover, it is not unreasonable to surmise that the pathogen may evolve in response to *Wolbachia* infection in mosquitoes, with potential adverse results. Oxitec believes that additional information and analysis is required regarding interactions of host insect, the pathogen, and *Wolbachia*, to ensure that *Wolbachia* does not ultimately select for a more dangerous pathogen [Brelsfoard and Dobson, 2009].

Horizontal transmission between unrelated host species is a proven phenomenon in *Wolbachia* [Baldo et al., 2008]. Studies have demonstrated that genetic sequences, ranging in size from single genes to entire bacterial genomes, have been transferred from *Wolbachia* to many of their insect hosts [Dunning Hotopp et al., 2007; Fenn et al., 2006; Hou et al., 2014; Kondo et al., 2002], and its effect on disease transmission is variable and potentially dangerous. We note that the Florida Keys has many national parks and protected species that could be susceptible to infection with *Wolbachia* with unknown consequences on the ecosystem. This is another reason that a full-fledged ecological risk assessment is appropriate prior to approval of the requested amendment and extension of the EUP.

There is evidence that male age and overcrowding during development (*i.e.*, under mass rearing conditions required to produce enough males for IIT to be effective) can reduce the cytoplasmic incompatibility effect in certain insects, rendering the males fertile [Yamada et al., 2007] and able to spread the *Wolbachia* infection through surviving females. Has this study been performed on *Aedes aegypti* to look at age effects and rearing conditions on the cytoplasmic incompatibility penetrance? If not, this should be performed as this is another potential route to simply spread a new *Wolbachia* species into the Florida Keys environment with unknown consequences.

EPA'S RESPONSE TO PUBLIC COMMENT #7

Due to controlled rearing conditions, no variation in the microbiome of *w*AlbB *Aedes aegypti* is expected. If the microbiome did inhibit the vertical transmission of *Wolbachia*, this would further lower the chances of a horizontal gene transfer (HGT) event or *w*AlbB *Aedes aegypti* establishment.

The action upon chromosome folding in *Wolbachia*-infected insects is documented and is adequately described for risk assessment purposes. Given the widespread infection of greater than 60% of insect species with *Wolbachia* and the number of different phenotypes associated with its presence, there may well be more than one mode of action depending on the host taxon infected and the strain of *Wolbachia* involved.

As discussed in Brelsfoard and Dobson (2009), in *Drosophila melanogaster*, the *w*Mel strain of *Wolbachia* may influence the susceptibility of this fly to RNA-type viruses. It is further hypothesized that this phenomenon may occur in other host species harboring *Wolbachia*; however, no direct evidence is provided. *Aedes aegypti* reared by UKDE for release of *Wolbachia*-infected males are checked for the presence of infectious virus particles as part of the manufacturing process. Any significant changes that may occur with respect to favoring the presence of a pathogen would therefore be noted as part of the quality assurance protocols in place and would need to be reported to EPA pursuant to FIFRA section 6(a)(2).

Oxitec, Ltd. is correct that HGT is a documented phenomenon recognized in transfer of genes between many species of prokaryotes to prokaryotes and prokaryotes to eukaryotes. It is also known in a few documented instances from *Wolbachia* to host organisms; however, this is not without controversy in that some of the sequences purportedly transferred from *Wolbachia* are quite possibly pseudogenes already present in the host insect or derived from non-*Wolbachia* sources. HGT is likely to occur over evolutionary time involving many species. Release of *Aedes aegypti* males on a small scale (as compared to the million plus species already harboring *Wolbachia* infections in the receiving environment) will not appreciably alter the quantitative measure of HGT phenomena during the course of population suppression of mosquitoes in a defined area. Further discussion of this phenomenon is contained in O'Neill (2016) and Dobson et al. (2016).

Calvitti et al. (2015) showed that *w*AlbA *Aedes albopictus* in dense rearing conditions did not decrease the cytoplasmic incompatibility (CI) effect. Islam and Dobson (2006) also showed that rearing *Aedes albopictus* with *Wolbachia* under crowded, low food conditions did not impact the CI effect. Yamada et al. (2007) cited by Oxitec, Ltd. refers to the effect in *Drosophila*, not mosquitoes.

PUBLIC COMMENT #8

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0015"] – On behalf of the Center for Food Safety (CFS) and our 750,000 members, I am writing to request a 30-day extension of the comment period to provide input on the University of Kentucky's Department of Entomology requesting an amendment and extension to an already existing experimental use permit (EUP) for *Wolbachia pipientis*, *w*AlbB Strain.

EPA is seeking to allow manufacturers to field test pesticides under development. Manufacturers are required to obtain a EUP before testing new pesticides or new uses of pesticides. The University of Kentucky has proposed to continue to field test a new strain of *Wolbachia pipientis* (*w*AlbB Strain) to determine its pesticidal value for suppression and elimination of *Aedes aegypti*, a mosquito that vectors some human diseases. According to the EPA, this EUP application may be of regional or national significance, and therefore is seeking public comment.

CFS does not believe that it has sufficient time to meaningfully review, analyze, and provide comments on a potential action of this magnitude. An extension to this unique field trial encompasses an array of complicated issues and requires careful review by the public, scientists, and other experts.

Therefore, CFS urges EPA to extend the comment period by at least 30 days.

[HYPERLINK "https://www.regulations.gov/document?D=EPA-HQ-OPP-2015-0374-0018"] – Consistent with EPA's FIFRA public transparency policy, EPA should allow a public comment period of at least 30 days prior to any approval of the EUP application 88877-EUP-2.

EPA'S RESPONSE TO PUBLIC COMMENT #8

The notice of receipt (NOR) for the initial application for experimental use permit 88877-EUP-2, which was published for comment in the Federal Register on July 29, 2015 ([HYPERLINK "https://www.gpo.gov/fdsys/pkg/FR-2015-07-29/pdf/2015-18615.pdf"]), received minimal comment. Nonetheless, in accordance with [HYPERLINK "http://www.ecfr.gov/cgi-bin/text-idx?SID=c302a420f9f97dbd953fdb5f33bf7dea&mc=true&node=se40.26.172_111&rgn=div8"], EPA published for comment in the Federal Register a notice of its receipt of the application for amendment and extension of experimental use permit 88877-EUP-2 on April 26, 2016 ([HYPERLINK "https://www.gpo.gov/fdsys/pkg/FR-2016-04-26/pdf/2016-09745.pdf"]), providing an overall summary of the request for public inspection that included information on the active ingredient, the use pattern, quantity of pesticide, total acreage, and locations of application. That NOR provided a 30-day comment period. As is typical for other receipt notices published for experimental use permits, the information made available to the public for UKDE's application was not extensive because EPA's review of the materials submitted by UKDE was still in progress.

The additional comment period "[c]onsistent with EPA's FIFRA public transparency policy" requested by Oxitec, Ltd. appears to refer to EPA's "Public Participation Process for Registration Actions" policy (*see* [HYPERLINK "https://www.epa.gov/pesticide-registration/public-participation-process-registration-actions"]). That policy is designed to allow EPA to be more transparent for certain registration actions (e.g., new active ingredients and first food uses) under FIFRA section 3 by providing the public a chance to comment at a time when comprehensive information and analyses are available, subsequent to the NOR required by section 3(c)(4) of FIFRA. The EUP amendment and extension request submitted by UKDE is a request to conduct limited testing under FIFRA section 5, which is not an action covered under EPA's Public Participation Process for Registration Actions policy.

For the reasons discussed immediately above, as well as in consideration of encouraging the development of pesticides that may be helpful in combating mosquitoes that vector diseases (e.g., Zika, dengue, and chikungunya viruses) detrimental to human health, EPA decided not to extend the comment

period on the NOR for the present EUP action or to open another comment period as is done for certain registration actions under the Public Participation Process for Registration Actions policy.

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